

Intramembrane proteolytic cleavage of a membrane-tethered transcription factor by a metalloprotease depends on ATP

Ruanbao Zhou^{a,b}, Christina Cusumano^a, Dexin Sui^a, R. Michael Garavito^a, and Lee Kroos^{a,1}

^aDepartment of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824; and ^bDepartment of Biology and Microbiology, South Dakota State University, Brookings, SD 57007

Edited by Richard M. Losick, Harvard University, Cambridge, MA, and approved July 20, 2009 (received for review February 12, 2009)

Regulated intramembrane proteolysis (RIP) involves cleavage of a transmembrane segment of a protein. RIP governs diverse processes in a wide variety of organisms and is carried out by different types of intramembrane proteases (IPs), including a large family of metalloproteases. The *Bacillus subtilis* SpoIVFB protein is a putative metalloprotease that cleaves membrane-tethered Pro- σ^K , releasing σ^K to direct transcription of genes necessary for spore formation. By attaching an extra transmembrane segment to the N terminus of SpoIVFB, expression in *E. coli* was improved more than 100-fold, facilitating purification and demonstration of metalloprotease activity, which accurately cleaved purified Pro- σ^K . Uniquely for IPs examined so far, SpoIVFB activity requires ATP, which binds to the C-terminal cystathionine- β -synthase (CBS) domain of SpoIVFB. Deleting just 10 residues from the C-terminal end of SpoIVFB nearly eliminated cleavage of coexpressed Pro- σ^K in *E. coli*. The CBS domain of SpoIVFB was shown to interact with Pro- σ^K and ATP changed the interaction, suggesting that ATP regulates substrate access to the active site and renders cleavage sensitive to the cellular energy level, which may be a general feature of CBS-domain-containing IPs. Incorporation of SpoIVFB into preformed liposomes stimulated its ability to cleave Pro- σ^K . Cleavage depended on ATP and the correct peptide bond was shown to be cleaved using a rapid and sensitive mass spectrometry assay. A system for biochemical studies of RIP by a metalloprotease in a membrane environment has been established using methods that might be applicable to other IPs.

Bacillus subtilis | intramembrane protease | regulated intramembrane proteolysis | SpoIVFB | sporulation

During the past decade, RIP emerged as an important mechanism controlling diverse processes in living organisms from bacteria to humans (1–4). Among the first IPs identified were human site-2 protease (S2P) (5) and a bacterial ortholog in *Bacillus subtilis* called SpoIVFB (6–9). These proteases are membrane-embedded, and mutational studies provided evidence that they are metalloproteases with active-site residues in transmembrane segments. Both cleave membrane-tethered transcription factors (MTTFs), with S2P releasing sterol regulatory element binding proteins or ATF6 from intracellular membranes in processes that respond to sterol signals or unfolded proteins, respectively (5, 10), and SpoIVFB releasing σ^K from the outer forespore membrane (OFM) (Fig. S1) in response to a signal generated from within the forespore during endospore formation (8, 9). σ^K binds to core RNA polymerase (RNAP) in the mother cell, which surrounds the forespore, and σ^K RNAP transcribes genes required to form the cortex and coat layers of the spore. Proteins with sequence similarity to S2P and SpoIVFB are involved in RIP events that control bacterial mating (11), stress responses (12–14), polar organelle biogenesis (15), cell division (16), and pathogenesis (17).

In addition to metalloproteases, two other types of IPs have been discovered. Rhomboids are a large family of serine proteases that play key roles in diverse processes (4). Presenilin is the catalytic subunit of γ -secretase, an aspartyl protease that cleaves the β -amy-

loid precursor protein implicated in Alzheimer's disease, as well as Notch receptor and other substrates that govern animal growth and development (2). Related to presenilins are signal peptide peptidases, which not only clear remnant signal peptides from membranes, but produce immune system signals and facilitate viral infection (2, 3).

The involvement of IPs in processes related to disease, and the fundamental question of how these enzymes catalyze peptide bond hydrolysis within the hydrophobic environment of lipid bilayers, has spurred interest in mechanistic and structural studies. Residues important for enzyme activity and substrate recognition have been identified by analysis of mutant alleles in vivo for many IPs and their substrates. Activity of detergent-solubilized, purified IPs established that the metalloprotease (18, 19), rhomboid (20–22), and signal peptide peptidase (23) types can function as single polypeptides, whereas presenilin requires three other polypeptides to form active γ -secretase (24). With the exception of the metalloprotease type, the other IP types have been reconstituted into artificial membranes, revealing that the lipid environment can influence IP activity (21, 25, 26). Crystal structures of detergent-solubilized, bacterial IPs, have revealed transmembrane segments arranged to form channels that presumably deliver water for peptide bond hydrolysis to active sites predicted to lie within the membrane (3). The structures suggest models for how substrates might gain access to active sites, with certain loops or transmembrane segments functioning as lateral gates (3).

B. subtilis SpoIVFB has been proposed to be a metalloprotease based on sequence similarity and mutational studies (8, 9), but direct evidence has been lacking. Coexpression of SpoIVFB and its putative substrate, Pro- σ^K , in *Escherichia coli* (Fig. S1) resulted in production of σ^K , providing additional evidence that SpoIVFB is an IP (7, 27), but SpoIVFB accumulated poorly. Here, we demonstrate that SpoIVFB can be expressed highly in *E. coli*. We show that purified SpoIVFB cleaves purified Pro- σ^K accurately in a reaction that depends on addition of zinc and is inhibited by the metal chelator 1,10-phenanthroline, providing direct evidence that SpoIVFB is a metalloprotease. Interestingly, the reaction depends on ATP, unlike other IPs examined so far. SpoIVFB belongs to a subfamily of putative metalloproteases with a C-terminal CBS domain (28). These domains have been shown to bind adenosine-containing ligands (29). We provide evidence that the CBS domain of SpoIVFB is important for activity in vivo, and that ATP binds to the purified CBS domain in vitro and affects its interaction with Pro- σ^K . We propose that ATP regulates Pro- σ^K access to the active

Author contributions: R.Z., C.C., D.S., R.M.G., and L.K. designed research; R.Z., C.C., and D.S. performed research; R.Z., C.C., and D.S. contributed new reagents/analytic tools; R.Z., C.C., R.M.G., and L.K. analyzed data; and R.Z., C.C., R.M.G., and L.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹To whom correspondence should be addressed. E-mail: kroos@msu.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0901455106/DCSupplemental.

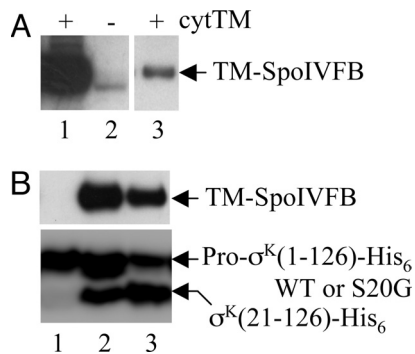


Fig. 1. Overexpression of SpoIVFB and Pro- σ^K in *E. coli*. (A) Accumulation of SpoIVFB with or without N-terminal cytTM. *E. coli* cells bearing pZR209 to produce cytTM-SpoIVFB-FLAG₂-His₆ (lanes 1 and 3) or pZR260 to produce SpoIVFB-FLAG₂-His₆ (lane 2) were collected 2 h after IPTG induction. Extracts from equivalent cell amounts (based on the OD₆₀₀ of the culture) (lanes 1 and 2) or 100-fold less extract (lane 3) were immunoblotted using antibodies against FLAG. (B) Cleavage of Pro- σ^K (1-126)-His₆ by TM-SpoIVFB. *E. coli* cells bearing pZR327 to produce Pro- σ^K (1-126)-His₆ S20G alone (lane 1) or in combination with pZR209 to also produce TM-SpoIVFB (lane 3), or bearing pZR12 to produce wild-type (WT) Pro- σ^K (1-126)-His₆ in combination with pZR209 (lane 2), were collected 2 h after IPTG induction. Extracts from equivalent cell amounts were immunoblotted using antibodies against FLAG (top panel) or penta-His (bottom panel).

site of SpoIVFB and that energy-sensing might be a general feature of CBS domain-containing IPs. Incorporation of SpoIVFB into artificial membranes stimulated its ability to cleave Pro- σ^K , and cleavage depended on ATP and was accurate, establishing that intramembrane proteolysis of an MTF by a metalloprotease can be reconstituted in vitro.

Results

Overexpression of *B. subtilis* SpoIVFB and Pro- σ^K in *E. coli* for Purification. Native SpoIVFB is expressed poorly in *E. coli*, making it difficult to purify for biochemical studies. Expression of some membrane proteins can be improved by fusion with a transmembrane segment (denoted cytTM, residues 1–23) from rabbit cytochrome P450 2B4 (30). The coding sequence for this segment was fused to the 5'-end of the *B. subtilis* spoIVFB gene in an expression vector designed to produce cytTM-SpoIVFB-FLAG₂-His₆ (hereafter referred to as TM-SpoIVFB), which also has two FLAG tags and six histidine residues fused to its C terminus to facilitate detection and purification (Fig. S1). Addition of cytTM to SpoIVFB-FLAG₂-His₆ increased its accumulation at least 100-fold (Fig. 1A). TM-SpoIVFB cleaved Pro- σ^K (1-126)-His₆ accurately to σ^K (21-126)-His₆ (Fig. 1B, lane 2), as shown previously for recombinant His₁₀-SpoIVFB-GFP expressed in *E. coli* (27), and as verified for TM-SpoIVFB by determining the N-terminal amino acid sequence (by Edman degradation) of the σ^K (21-126)-His₆ product. TM-SpoIVFB also accurately cleaved Pro- σ^K (1-126)-His₆ with a substitution of glycine for serine at residue 20 (S20G) immediately preceding the cleavage site, based on the N-terminal amino acid sequence of the product, and cleavage of this substrate was enhanced relative to the wild-type substrate (Fig. 1B, lane 3). Therefore, we purified Pro- σ^K (1-126)-His₆ S20G (designated Pro- σ^K S20G hereafter) and TM-SpoIVFB for biochemical studies.

Activity of several different IPs has been observed in vitro in the presence of the nonionic detergents *n*-dodecyl- β -D-maltoside (DDM) or *n*-decyl- β -D-maltoside (DM) (18–23). DDM and DM were ineffective at solubilizing TM-SpoIVFB from membranes as compared with sodium dodecanoyl sarcosine (sarkosyl), so the latter was used for initial solubilization, followed by a switch to DM during purification. TM-SpoIVFB

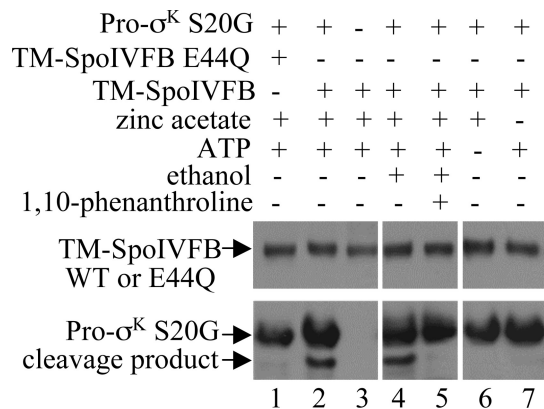


Fig. 2. Cleavage of Pro- σ^K S20G by TM-SpoIVFB in vitro requires zinc and ATP. Reaction mixtures contained the indicated components, and after incubation, were immunoblotted using antibodies against FLAG (top panel) or penta-His (bottom panel). Lane 3 shows a control reaction mixture that contained only TM-SpoIVFB. Lane 4 shows a control for lane 5, since 1,10-phenanthroline was dissolved in ethanol.

was purified by cobalt-affinity chromatography followed by gel filtration (Fig. S2A). As a negative control, a mutant form of TM-SpoIVFB with a substitution of glutamine for glutamate at residue 44 (E44Q) was purified in parallel (Fig. S2A). The E44Q substitution is hypothesized to inactivate the putative metalloprotease by preventing activation of a water molecule for peptide bond hydrolysis (8, 9). A similar approach was used to purify Pro- σ^K S20G except it was solubilized from membranes with a combination of sarkosyl and DDM, followed by a switch to DDM alone during purification (Fig. S2B).

Cleavage of Pro- σ^K S20G by TM-SpoIVFB in Vitro. To test for cleavage of Pro- σ^K S20G by TM-SpoIVFB in vitro, the proteins were incubated together in the presence of DM (0.1%) and DDM (0.015%). TM-SpoIVFB cleaved Pro- σ^K S20G in a reaction mixture that also contained zinc acetate and ATP (Fig. 2, lane 2), but TM-SpoIVFB E44Q did not cleave Pro- σ^K S20G (Fig. 2, lane 1), providing direct evidence that SpoIVFB is the protease that cleaves Pro- σ^K , and supporting the idea that E44, as part of the HEXXH motif found in metalloproteases (7), plays a crucial role in catalysis (7–9). As expected for a metalloprotease, TM-SpoIVFB cleavage of Pro- σ^K S20G was inhibited by the metal chelator 1,10-phenanthroline (Fig. 2, lane 5). Omission of zinc acetate from the reaction mixture prevented cleavage (Fig. 2, lane 7), suggesting that TM-SpoIVFB becomes zinc-depleted during purification. Interestingly, and uniquely for intramembrane proteases described so far, cleavage of Pro- σ^K S20G by TM-SpoIVFB was dependent on ATP (Fig. 2, lane 6). Neither ADP nor GTP had the strong stimulatory effect of ATP. Taken together, these results show that SpoIVFB is a zinc- and ATP-dependent metalloprotease that cleaves Pro- σ^K .

When wild-type Pro- σ^K (1-126)-His₆ lacking the S20G substitution was purified as described for Pro- σ^K S20G and incubated with TM-SpoIVFB as described above, cleavage was barely detectable, consistent with the enhanced cleavage of Pro- σ^K S20G relative to the wild-type substrate in vivo (Fig. 1B).

Role of ATP and the CBS Domain of SpoIVFB in Cleavage of Pro- σ^K . Since SpoIVFB belongs to a subfamily of putative metalloproteases with a C-terminal CBS domain (28) and since these domains have been shown to bind adenosine-containing ligands (29), we hypothesized that the ATP dependence of Pro- σ^K cleavage by TM-SpoIVFB in vitro reflects an important role of the CBS domain in binding ATP. To test the importance of the

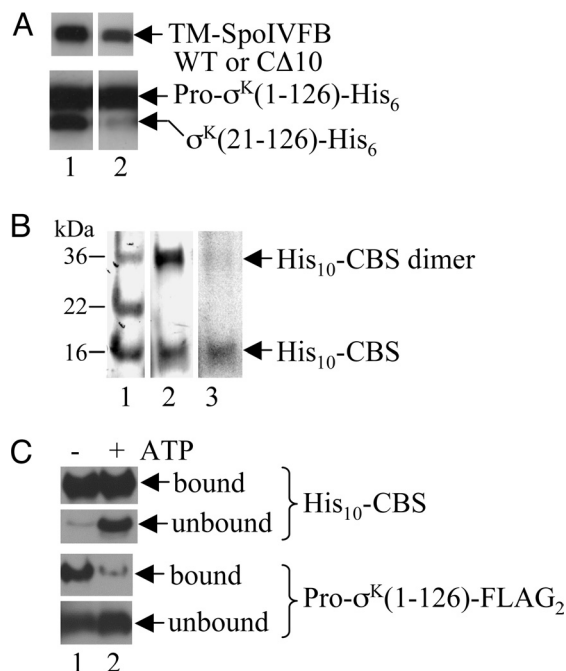


Fig. 3. Role of ATP and the CBS domain of SpoIVFB in cleavage of Pro- σ^K . (A) The C-terminal 10 residues of SpoIVFB are important for cleavage of Pro- σ^K . *E. coli* cells bearing pZR12 to produce Pro- σ^K (1–126)-His₆ and either pZR209 to produce wild-type (WT) TM-SpoIVFB (lane 1) or pZR244 to produce mutant TM-SpoIVFB lacking the C-terminal 10 residues (Δ10) (lane 2), were collected 2 h after IPTG induction. Extracts from equivalent cell amounts were immunoblotted using antibodies against FLAG (top panel) or penta-His (bottom panel). (B) The CBS domain of SpoIVFB binds ATP. The Immobilon-P membrane was subjected to autoradiography to detect [α -³²P]ATP bound to protein (lane 3), then stained with Coomassie blue to detect His₁₀-CBS (lane 2) and marker proteins (lane 1). (C) ATP affects the interaction of Pro- σ^K with the CBS domain of SpoIVFB. Cobalt-affinity chromatography of His₁₀-CBS and coexpressed Pro- σ^K (1–126)-FLAG₂ in the absence (lane 1) or presence (lane 2) of ATP as indicated with the flow-through (unbound) and high imidazole eluate (bound) immunoblotted using antibodies against penta-His and FLAG to detect His₁₀-CBS and Pro- σ^K (1–126)-FLAG₂, respectively.

CBS domain in vivo, we deleted the coding sequence for the entire domain (residues 204 to 288) from the *spoIVFB* gene in the expression vector designed to produce TM-SpoIVFB. Upon coexpression in *E. coli* with Pro- σ^K S20G, very little or no cleavage was observed, but TM-SpoIVFB lacking the CBS domain accumulated poorly (Fig. S3). Based on genetic studies (31), it appeared that deletion of 10 residues from the C-terminal end of SpoIVFB might render it inactive. Indeed, deletion of residues 279 to 288 from TM-SpoIVFB nearly eliminated cleavage of coexpressed Pro- σ^K S20G in *E. coli*, while only slightly impairing accumulation of the enzyme (Fig. 3A). We conclude that the CBS domain of TM-SpoIVFB is important for the protein to accumulate in *E. coli* and that the C-terminal 10 residues of this domain are important for activity in vivo.

To test whether the CBS domain of SpoIVFB binds ATP, we expressed the N-terminally histidine-tagged domain (His₁₀-CBS) in *E. coli* for purification. Surprisingly, His₁₀-CBS was predominantly membrane-associated, so membranes were isolated and His₁₀-CBS was solubilized with a combination of sarkosyl and DDM, followed by a switch to DDM alone during purification using cobalt-affinity chromatography (Fig. S2C). Purified His₁₀-CBS was incubated with [α -³²P]ATP, then the sample was mixed with SDS/PAGE sample buffer, but it was not boiled before SDS/PAGE and transfer to an Immobilon-P membrane. The membrane was subjected to autoradiography and then stained with Coomassie blue to detect labeled ATP that had bound to protein. Under these conditions of incom-

plete denaturation, His₁₀-CBS migrated as two bands at positions expected for monomer and dimer. The two bands contained similar amounts of protein based on the Coomassie blue staining intensity (Fig. 3B, lane 2), but the lower band bound more ATP (Fig. 3B, lane 3), indicating that the CBS domain monomer binds ATP specifically.

Since His₁₀-CBS was predominantly membrane-associated in *E. coli*, we tested whether it interacts with coexpressed Pro- σ^K . For this experiment, we constructed Pro- σ^K (1–126) with two C-terminal FLAG tags [Pro- σ^K (1–126)-FLAG₂] for detection by immunoblot. This protein was coexpressed with His₁₀-CBS in *E. coli*, the cells were lysed and membranes were isolated and solubilized with sarkosyl, and His₁₀-CBS complexes were purified using cobalt-affinity chromatography. Beginning at the cell lysis step, ATP (1 mM) was added to or omitted from all buffers. In the absence of ATP, a portion of the Pro- σ^K (1–126)-FLAG₂ co-purified with His₁₀-CBS bound to cobalt beads (Fig. 3C, lane 1). Strikingly, in the presence of ATP, a portion of the His₁₀-CBS failed to bind to the cobalt beads and very little Pro- σ^K (1–126)-FLAG₂ was observed in the bound fraction (Fig. 3C, lane 2). Controls showed that ATP did not inhibit binding of His₁₀-CBS to cobalt in the absence of Pro- σ^K (1–126)-FLAG₂, and as expected, very little Pro- σ^K (1–126)-FLAG₂ bound to cobalt in the presence or absence of ATP. These results show that Pro- σ^K (1–126)-FLAG₂ interacts with His₁₀-CBS and that ATP affects the interaction in a way that inhibits His₁₀-CBS and Pro- σ^K (1–126)-FLAG₂ binding to cobalt beads. Taken together, our results suggest that the role of ATP is to change the interaction between Pro- σ^K and the CBS domain of SpoIVFB in a way that leads to cleavage.

Incorporation of TM-SpoIVFB and Pro- σ^K S20G into Preformed Liposomes. To our knowledge, no purified IP of the metalloprotease type has been incorporated into liposomes and shown to cleave its substrate. To demonstrate that a metalloprotease can function within membranes and to establish methods for studying the effects of lipids and inhibitors in vitro, we formed liposomes from *E. coli* lipids and tested whether TM-SpoIVFB and Pro- σ^K S20G were incorporated into liposomes upon detergent removal. Liposomes were formed in the presence of green pyranine dye so that green proteoliposomes could be visualized during subsequent gel filtration chromatography (Fig. S4A). Both TM-SpoIVFB and Pro- σ^K S20G were incorporated into preformed liposomes upon slow removal of detergent by gradual addition of Bio-SM2 beads. Green proteoliposomes eluted at the void volume of the gel filtration column and were shown by immunoblot to contain TM-SpoIVFB (Fig. S4B) or Pro- σ^K S20G (Fig. S4D). The proteoliposomes failed to form in the absence of *E. coli* lipids. TM-SpoIVFB that had been incorporated into liposomes was sedimented by high-speed centrifugation, unless the proteoliposomes were treated with detergent to solubilize the TM-SpoIVFB (Fig. S4C). As a further control, σ^K (21–126)-His₆ was shown not to incorporate into liposomes, while Pro- σ^K S20G that had been incorporated was sedimented by high-speed centrifugation, and after treatment of these proteoliposomes with detergent, Pro- σ^K S20G remained in the supernatant upon centrifugation (Fig. S4E). We conclude that both TM-SpoIVFB and Pro- σ^K S20G can be independently incorporated into liposomes formed from *E. coli* lipids.

Cleavage of Pro- σ^K S20G by TM-SpoIVFB Proteoliposomes. To test the ability of TM-SpoIVFB proteoliposomes to cleave Pro- σ^K S20G, we performed three types of experiments. In the first type of experiment, TM-SpoIVFB proteoliposomes and Pro- σ^K S20G proteoliposomes prepared separately as described above were mixed in the presence of zinc and ATP. To some samples, cobalt beads were added, because the C-terminal His₆ tags present on both TM-SpoIVFB and Pro- σ^K S20G were expected to bind to cobalt on the beads, possibly bringing the enzyme and substrate together in close proximity. Such an effect has been reported

the protein translocon (34). By selecting an N-terminal transmembrane segment that is efficiently recognized and inserted into the *E. coli* inner membrane, a TM-fusion construct might be created that increases the level of properly incorporated target membrane protein. We selected the N-terminal transmembrane segments from three different membrane proteins that are readily incorporated into the *E. coli* inner membrane such that a very high yield (>10 mg purified protein per liter culture) is achieved (30, 35). The coding sequences for the three transmembrane segments (including cytTM) were cloned and used to create sets of expression vectors. The optimized expression construct used in this study yields TM-SpoIVFB in which cytTM is predicted to be oriented with its N terminus in the periplasm of *E. coli* (Fig. S1). Addition of cytTM to the N terminus of SpoIVFB-FLAG₂-His₆ increased its accumulation in *E. coli* at least 100-fold upon induction using a T7 RNAP system (Fig. 1A). The success of the TM-fusion in increasing the yield of SpoIVFB begs the question whether TM-fusions with other IPs might enhance their accumulation in *E. coli* to allow biochemical and structural analyses.

Activity of SpoIVFB in vitro was dependent on the addition of zinc and was inhibited by addition of the metal chelator 1,10-phenanthroline. Inhibition by 1,10-phenanthroline was observed for purified *E. coli* RseP (also known as YaeL) (19) and a C-terminally-truncated fragment of a *Methanocaldococcus jannaschii* S2P homolog (mjS2P) (18), two metalloprotease-type IPs whose activity has been observed in the presence of detergents. Dependence of RseP on zinc was not tested, but 5 μ M zinc acetate was included in reactions (19). In the case of mjS2P, zinc addition to reactions was not necessary to observe protease activity, and zinc was bound to the protein fragment in an \approx approximate 1:1 molar ratio (18). Unlike RseP and mjS2P expressed in *E. coli*, which were solubilized from membranes by 1% DDM or 1% DM, respectively, TM-SpoIVFB was not efficiently solubilized by these non-ionic detergents, but was efficiently solubilized by 1% sarkosyl, an ionic detergent. It is possible that zinc is lost from TM-SpoIVFB during solubilization or a subsequent purification step, necessitating addition of zinc to the in vitro reaction.

In contrast to IPs studied previously, we discovered that SpoIVFB requires ATP to cleave its substrate. Our results suggest that the role of ATP is to bind to the CBS domain of SpoIVFB and change its interaction with Pro- σ^K in a way that leads to cleavage. Binding of ATP and Pro- σ^K to the N-terminally histidine-tagged CBS domain appeared to inhibit binding of the complex to cobalt beads (Fig. 3C). The N-terminal end of the CBS domain follows transmembrane segment 6 of SpoIVFB, which has been proposed to be involved in regulating substrate access to the active site by functioning as a lateral gate (18), so we propose that ATP changes the conformation of the SpoIVFB/Pro- σ^K complex to open the gate (Fig. S6). In addition, ATP might influence SpoIVFB oligomerization since the CBS domain monomer bound more ATP than the dimer (Fig. 3B). During gel filtration chromatography (in the absence of ATP), the peak of detergent-solubilized TM-SpoIVFB eluted at \approx approximately 320 kDa, consistent with a tetramer if associated detergent roughly doubles the molecular weight. Perhaps ATP facilitates both SpoIVFB monomer formation and its productive interaction with substrate. CBS domains have been shown to bind adenosine-containing ligands such as ATP, ADP, AMP, and/or S-adenosylmethionine, and appear to function as sensors of cellular energy status that regulate the activity of metabolic enzymes, kinases, and channels (29). By analogy, we propose that the CBS domain of SpoIVFB regulates its activity in response to ATP, serving as an energy-sensing switch or rheostat that governs expression the σ^K regulon. The CBS domain of SpoIVFB is exposed to the mother cell cytoplasm, but recent studies suggest that metabolites like ATP are shared

through channels that form between the mother cell and the forespore (36, 37), so activation of the σ^K regulon could be coupled to the overall energy status of the developing organism.

SpoIVFB is representative of a subfamily of putative metalloprotease-type IPs with one or more C-terminal CBS domains (28). Subfamily members are found in bacteria, archaea, and plants. CBS domains typically occur as tandem pairs that associate to form a Bateman domain (38, 39). Interestingly, full-length mjS2P is predicted to have a C-terminal Bateman domain, but this part of the protein was removed from the fragment whose structure was determined (18). Activity of C-terminally-truncated mjS2P in vitro did not require addition of ATP to cleave an artificial protein substrate (18), so it remains an open question whether full-length mjS2P would require ATP to cleave a natural substrate. *E. coli* RseP is not predicted to have a CBS domain and the purified enzyme did not require addition of ATP to cleave its natural substrate (19). *B. subtilis* SpoIVFB and its orthologs in *Bacilli* are predicted to have a single C-terminal CBS domain. We speculate that energy sensing is a general characteristic of CBS-domain-containing IPs.

Our effort to reconstitute intramembrane proteolysis of Pro- σ^K by SpoIVFB in vitro was facilitated by our finding that Pro- σ^K S20G is more readily cleaved than the wild-type substrate. We hypothesized that a residue with a small side chain at the position preceding the cleavage site might improve access to the target peptide bond. Indeed, the S20G substitution in Pro- σ^K increased cleavage upon coexpression with SpoIVFB in *E. coli* (Fig. 1B). Moreover, Pro- σ^K S20G was a better substrate for purified TM-SpoIVFB and for TM-SpoIVFB proteoliposomes (Fig. 4C). The results with purified enzyme indicate that a membrane environment is not required for better cleavage of the mutant substrate, suggesting that the S20G substitution changes the interaction of Pro- σ^K with SpoIVFB rather than changing its interaction with membranes. The rhomboid IP, GlpG of *E. coli*, also prefers a residue with a small side chain at the position preceding the cleavage site (40), so this could be a broadly-conserved characteristic of IPs. Our results demonstrate that Pro- σ^K can interact directly with an artificial membrane. Previous studies showed that residues 1–27 of Pro- σ^K are sufficient for membrane localization of a Pro- σ^K -GFP chimera in *B. subtilis* (41) and that Pro- σ^K is predominantly membrane-associated when expressed in *E. coli* (27). However, the findings that Pro- σ^K could be partially solubilized from membranes by salt treatment and that Pro- σ^K partitioned to the aqueous phase of a Triton X-114 fractionation experiment suggested that the pro-sequence might not insert into a membrane like a typical transmembrane segment or that Pro- σ^K might interact with an unidentified protein in the membrane (42). The latter model appears to be incorrect since Pro- σ^K S20G associates readily with preformed liposomes made using *E. coli* lipids (Fig. S4). Whether the pro-sequence spans the membrane as depicted in Fig. S1 or interacts differently with the membrane is unknown, but clearly Pro- σ^K is an MTTF.

Incorporation of SpoIVFB into liposomes stimulated its ability to cleave Pro- σ^K . The liposomes were formed from *E. coli* lipids in this study, but the establishment of a proteoliposome system opens the door for exploration of lipid effects on a metalloprotease-type IP. Particular lipids or lipid mixtures dramatically increase or decrease activity of other types of IPs (21, 25, 26). SpoIVFB normally functions in the OFM during *B. subtilis* sporulation (Fig. S1) (43). The OFM is derived from the mother cell membrane and although its lipid composition is unknown, it very likely differs greatly from *E. coli* lipids. Knowledge about the lipid environment that favors SpoIVFB activity should facilitate efforts toward crystallization and structure determination. Reconstitution into proteoliposomes also allows a more realistic assessment of potential IP inhibitors (26) and rapid, sensitive quantification of cleavage by mass spectrometry, an assay amenable to high-throughput screening. SpoIVFB is inhib-

ited by a small, membrane protein, BofA, which has been proposed to provide a fourth zinc ligand in a complex that also includes the SpoIVFA membrane protein (27). Cleavage of SpoIVFA and BofA by serine proteases secreted from the forespore into the space between the inner forespore membrane (IFM) and OFM (Fig. S1) appears to relieve inhibition of SpoIVFB (44–46). While reconstruction of this entire signal transduction pathway remains a daunting challenge, the reconstitution of active SpoIVFB proteoliposomes described herein should facilitate mechanistic studies of the natural inhibitor BofA and screening for artificial inhibitors. The insights gained from this study and the methods developed may be applicable to other IPs, some of which are potential targets for therapeutic intervention (1, 17).

Materials and Methods

Construction of Plasmids. Plasmid descriptions and details of their construction are in Table S1. Sequences of oligonucleotides are in Table S2. Mutations were introduced into sigK or spoIVFB using the QuikChange site-directed mutagenesis kit (Stratagene). All cloned PCR products and all mutagenized genes were subjected to DNA sequencing to confirm the presence of the desired sequences.

Small-Scale Overexpression of Proteins and Immunoblots. Plasmids were transformed into *E. coli* BL21(DE3) (Novagen) and strains were grown and induced with IPTG to overexpress proteins as described (27). Whole-cell extracts were prepared and immunoblots were performed as described (27). Horseradish peroxidase-conjugated antibodies against penta-His (Qiagen) and against FLAG (Sigma) were used at 1:5,000 dilution.

1. Brown MS, Ye J, Rawson RB, Goldstein JL (2000) Regulated intramembrane proteolysis: A control mechanism conserved from bacteria to humans. *Cell* 100:391–398.
2. Wolfe MS, Kopan R (2004) Intramembrane proteolysis: Theme and variations. *Science* 305:1119–1123.
3. Urban S, Shi Y (2008) Core principles of intramembrane proteolysis: Comparison of rhomboid and site-2 family proteases. *Curr Opin Struct Biol* 18:432–441.
4. Freeman M (2008) Rhomboid proteases and their biological functions. *Annu Rev Genet* 42:191–210.
5. Rawson R, et al. (1997) Complementation cloning of SP2, a gene encoding a putative metalloprotease required for intramembrane cleavage of SREBPs. *Mol Cell* 1:47–57.
6. Cutting S, et al. (1990) A forespore checkpoint for mother-cell gene expression during development in *Bacillus subtilis*. *Cell* 62:239–250.
7. Lu S, Cutting S, Kroos L (1995) Sporulation protein SpoIVFB from *Bacillus subtilis* enhances processing of the sigma factor precursor pro- σ^K in the absence of other sporulation gene products. *J Bacteriol* 177:1082–1085.
8. Rudner D, Fawcett P, Losick R (1999) A family of membrane-embedded metalloproteases involved in regulated proteolysis of membrane-associated transcription factors. *Proc Natl Acad Sci USA* 96:14765–14770.
9. Yu, Y-TN, Kroos L (2000) Evidence that SpoIVFB is a novel type of membrane metalloprotease governing intercompartmental communication during *Bacillus subtilis* sporulation. *J Bacteriol* 182:3305–3309.
10. Ye J, et al. (2000) ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol Cell* 6:1355–1364.
11. An FY, Sulavik MC, Clewell DB (1999) Identification and characterization of a determinant (eep) on the *Enterococcus faecalis* chromosome that is involved in production of the peptide sex pheromone CAD1. *J Bacteriol* 181:5915–5921.
12. Kanehara K, Ito K, Akiyama Y (2002) Yael (EcfE) activates the σ^E pathway of stress response through a site-2 cleavage of anti- σ^E , RseA. *Genes Dev* 16:2147–2155.
13. Alba BM, Leeds JA, Onufryk C, Lu CZ, Gross CA (2002) DegS and Yael participate sequentially in the cleavage of RseA to activate the σ^E -dependent extracytoplasmic stress response. *Genes Dev* 16:2156–2168.
14. Schobel S, Zellmeier S, Schumann W, Wiegert T (2004) The *Bacillus subtilis* σ^{W} anti-sigma factor RsiW is degraded by intramembrane proteolysis through YluC. *Mol Microbiol* 52:1091–1105.
15. Chen JC, Viollier PH, Shapiro L (2005) A membrane metalloprotease participates in the sequential degradation of a *Caulobacter* polarity determinant. *Mol Microbiol* 55:1085–1103.
16. Bramkamp M, Weston L, Daniel RA, Errington J (2006) Regulated intramembrane proteolysis of FtsL protein and the control of cell division in *Bacillus subtilis*. *Mol Microbiol* 62:580–591.
17. Urban S (2009) Making the cut: Central roles of intramembrane proteolysis in pathogenic microorganisms. *Nat Rev Microbiol* 7:411–423.
18. Feng L, et al. (2007) Structure of a site-2 protease family intramembrane metalloprotease. *Science* 318:1608–1612.
19. Akiyama Y, Kanehara K, Ito K (2004) RseP (Yael), an *Escherichia coli* RIP protease, cleaves transmembrane sequences. *EMBO J* 23:4434–4442.
20. Lemberg MK, et al. (2005) Mechanism of intramembrane proteolysis investigated with purified rhomboid proteases. *EMBO J* 24:464–472.
21. Urban S, Wolfe MS (2005) Reconstitution of intramembrane proteolysis in vitro reveals that pure rhomboid is sufficient for catalysis and specificity. *Proc Natl Acad Sci USA* 102:1883–1888.
22. Maegawa S, Ito K, Akiyama Y (2005) Proteolytic action of GlpG, a rhomboid protease in the *Escherichia coli* cytoplasmic membrane. *Biochemistry* 44:13543–13552.
23. Sato T, et al. (2006) Signal peptide peptidase: Biochemical properties and modulation by nonsteroidal antiinflammatory drugs. *Biochemistry* 45:8649–8656.

Large-Scale Overexpression of Proteins for Purification. Overexpression of proteins, membrane isolation and solubilization, and protein purification are described in the *SI Materials and Methods*.

Reaction Mixtures with Purified Proteins. Reaction mixtures for in vitro proteolysis and ATP binding are described in the *SI Materials and Methods*.

Interaction of Pro- σ^K with the CBS Domain of SpoIVFB. Overexpression of proteins, membrane isolation and solubilization, and cobalt-affinity chromatography are described in the *SI Materials and Methods*.

Liposome Experiments. The preparation of liposomes, incorporation of proteins into liposomes, and reaction mixtures with proteoliposomes are described in the *SI Materials and Methods*.

Mass Spectrometry. MALDI-TOF analysis was performed as described in the *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank L. Alexander and R. Mark Worden of the Michigan State University Protein Expression Lab for performing the large-scale overexpression of TM-SpoIVFB; E. Lund of the Michigan State University Mass Spectrometry Facility for performing MALDI-TOF analysis; N. Kim, Y. Zheng, A. Scharmen, and M. Kroos for technical assistance; and R. Losick, B. Henry, D. Jones, and S. Ferguson-Miller for helpful comments on the manuscript. This research was supported by National Institutes of Health Grant GM43585 (to L.K.), Michigan State University Grant REF03-016 (to R.M.G.), the Michigan Agricultural Experiment Station, and the State of South Dakota Center for Bioprocessing Research and Development.

24. Sato T, et al. (2007) Active gamma-secretase complexes contain only one of each component. *J Biol Chem* 282:33985–33993.
25. Narayanan S, Sato T, Wolfe MS (2007) A C-terminal region of signal peptide peptidase defines a functional domain for intramembrane aspartic protease catalysis. *J Biol Chem* 282:20172–20179.
26. Osenkowski P, Ye W, Wang R, Wolfe MS, Selkoe DJ (2008) Direct and potent regulation of gamma-secretase by its lipid microenvironment. *J Biol Chem* 283:22529–22540.
27. Zhou R, Kroos L (2004) BofA protein inhibits intramembrane proteolysis of pro- σ^K in an intercompartmental signaling pathway during *Bacillus subtilis* sporulation. *Proc Natl Acad Sci USA* 101:6385–6390.
28. Kinch LN, Ginalski K, Grishin NV (2006) Site-2 protease regulated intramembrane proteolysis: Sequence homologs suggest an ancient signaling cascade. *Protein Sci* 15:84–93.
29. Scott JW, et al. (2004) CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J Clin Invest* 113:274–284.
30. Saribas AS, Gruenke L, Waskell L (2001) Overexpression and purification of the membrane-bound cytochrome P450 2B4. *Protein Expr Purif* 21:303–309.
31. Cutting S, Roels S, Losick R (1991) Sporulation operon *spoIVF* and the characterization of mutations that uncouple mother-cell from forespore gene expression in *Bacillus subtilis*. *J Mol Biol* 221:1237–1256.
32. Walsh NP, Alba BM, Bose B, Gross CA, Sauer RT (2003) OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. *Cell* 113:61–71.
33. Kroos L, Kunkel B, Losick R (1989) Switch protein alters specificity of RNA polymerase containing a compartment-specific sigma factor. *Science* 243:526–529.
34. Hessa T, et al. (2005) Recognition of transmembrane helices by the endoplasmic reticulum translocator. *Nature* 433:377–381.
35. Miroux B, Walker JE (1996) Over-production of proteins in *Escherichia coli*: Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* 260:289–298.
36. Meisner J, Wang X, Serrano M, Henriques AO, Moran CP, Jr (2008) A channel connecting the mother cell and forespore during bacterial endospore formation. *Proc Natl Acad Sci USA* 105:15100–15105.
37. Camp AH, Losick R (2009) A feeding-tube model for activation of a cell-specific transcription factor during sporulation in *Bacillus subtilis*. *Genes Dev* 23:1014–1024.
38. Bateman A (1997) The structure of a domain common to archaeobacteria and the homocystinuria disease protein. *Trends Biochem Sci* 22:12–13.
39. Ignoul S, Eggermont J (2005) CBS domains: Structure, function, and pathology in human proteins. *Am J Physiol Cell Physiol* 289:C1369–C1378.
40. Akiyama Y, Maegawa S (2007) Sequence features of substrates required for cleavage by GlpG, an *Escherichia coli* rhomboid protease. *Mol Microbiol* 64:1028–1037.
41. Prince H, Zhou R, Kroos L (2005) Substrate requirements for regulated intramembrane proteolysis of *Bacillus subtilis* pro- σ^K . *J Bacteriol* 187:961–971.
42. Zhang B, Hofmeister A, Kroos L (1998) The pro-sequence of pro- σ^K promotes membrane association and inhibits RNA polymerase core binding. *J Bacteriol* 180:2434–2441.
43. Resnekov O, Alper S, Losick R (1996) Subcellular localization of proteins governing the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. *Genes Cells* 1:529–542.
44. Dong TC, Cutting SM (2003) SpoIVB-mediated cleavage of SpoIVFA could provide the intercellular signal to activate processing of Pro- σ^K in *Bacillus subtilis*. *Mol Microbiol* 49:1425–1434.
45. Zhou R, Kroos L (2005) Serine proteases from two cell types target different components of a complex that governs regulated intramembrane proteolysis of pro- σ^K during *Bacillus subtilis* development. *Mol Microbiol* 58:835–846.
46. Campo N, Rudner DZ (2006) A branched pathway governing the activation of a developmental transcription factor by regulated intramembrane proteolysis. *Mol Cell* 23:25–35.